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Enzymatic and immunological properties of *Bungarus flaviceps* (red-headed krait) venom

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ABSTRACT: *Bungarus flaviceps* (red-headed krait) venom presents an intravenous LD_{50} of 0.32 µg/g and exhibits enzymatic activities similar to other *Bungarus* toxins. ELISA cross-reactions between anti-*Bungarus flaviceps* and a variety of elapid and viperid venoms were observed in the current study. Double-sandwich ELISA was highly specific, since anti-*B. flaviceps* serum did not cross-react with any tested venom, indicating that this assay can be used for species diagnosis in *B. flaviceps* bites. In the indirect ELISA, anti-*B. flaviceps* serum cross-reacted moderately with three different *Bungarus* venoms (9-18%) and *Notechis scutatus* venom, but minimally with other elapid and viperid toxins. The results indicated that *B. flaviceps* venom shares common epitopes with other *Bungarus* species as well as with *N. scutatus*. The lethality of the *B. flaviceps* venom was neutralized effectively by antiserum prepared against *B. candidus* and *B. flaviceps* toxins and a commercial bivalent elapid antivenom prepared against *B. multicinctus* and *Naja naja atra* venoms, but was not neutralized by commercial antivenoms prepared against Thai cobra, king cobra and banded krait. These data also suggested that the major lethal toxins of *B. flaviceps* venom are similar to those found in *B. multicinctus* and *B. candidus* venoms.

KEY WORDS: *Bungarus flaviceps* venom, enzymes, ELISA, neutralization.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Bungarus flaviceps (red-headed krait) is a rare snake found in Southeast Asia. It presents a very striking and distinctive coloration – namely a bright red head and tail with a black body that includes a low-lateral narrow bluish white stripe (Figure 1). The snake occurs in Burma, Thailand, Vietnam, Malaysia and Indonesia (1). The major lethal toxin having been isolated and cloned from *Bungarus flaviceps* is considered a novel isoform of β-bungarotoxin (2, 3). Its venom also contains a novel postsynaptic neurotoxin, termed κ-flavitoxin, which is a potent inhibitor of nicotinic transmission in autonomic ganglia (4, 5). The venoms of several common *Bungarus* species have been well investigated (6-8). Little, however, is known about the enzymatic properties of the *Bungarus flaviceps* venom. Chanhome *et al.* (9) reported that a commercial *Bungarus fasciatus* antivenom could neutralize the lethal toxicity of *B. flaviceps* venom. The immunological cross-reactivity of this venom, however, has not been investigated. We report herein a preliminary study on the enzymatic activities of *B. flaviceps* venom and its immunological cross-reactivities using enzyme-linked immunosorbent assay (ELISA).

Figure 1. An adult *Bungarus flaviceps* snake. The snake was approximately 6 feet in length; the head and tail were bright red while the body was black with a narrow bluish white stripe low on its side.

Bungarus flaviceps venom was obtained from a single milking of an adult individual (Figure 1) captured in central Malaysia. Venom yield was 50 mg dry weight. Other types of snake venoms used in this study were obtained from the Miami

Serpentarium Laboratories (USA), Latoxan (France) and Venom Supplies (Australia). All reagents, enzyme substrates and chemicals were purchased from Sigma Chemical Company (USA) or Bio-Rad Laboratories (USA). Monovalent antivenoms against *Ophiophagus hannah* (king cobra), *Naja kaouthia* (Thai cobra) and *Bungarus fasciatus* (banded krait) were obtained from the Thai Red Cross Society (TRCS), Bangkok, Thailand. Bivalent elapid antivenom (NIPM elapid antivenom, prepared against *Bungarus multicinctus* and *Naja naja atra*) was obtained from the National Institute of Preventive Medicine (NIPM), Taipei, Taiwan.

Lethality, procoagulant and anticoagulant, hemorrhagic and enzymatic activities were determined as previously described (7, 10). Monospecific rabbit antisera against *Bungarus flaviceps*, *Bungarus fasciatus*, *Bungarus candidus* (Malayan krait) and *Notechis scutatus* (Eastern tiger snake) were prepared using a hyperimmunization scheme (11). IgG antibodies and IgG-horseradish peroxidase conjugate were prepared according to Hudson and Hay (12) and Tijssen (13), respectively.

The indirect ELISA and double sandwich procedures were performed according to a previous description (14). The LD_{50} value was calculated according to Weil (15). To determine the neutralization effect of the antiserum/antivenom, a quantity equivalent to 3.0 LD_{50} of the venom, in 0.05 mL of saline solution, was mixed with 0.1-0.2 mL of antiserum (with appropriate dilution) and incubated for 30 minutes at 37^{o}C. After centrifugation, the supernatant was injected intravenously into the tail vein of ICR mice. Mortality after 24 hours was determined (n = 6). The rabbits and mice were supplied by the Central Animal House, School of Medicine, University of Malaya, Kuala Lumpur, Malaysia.

The intravenous LD_{50} of the *B. flaviceps* venom was determined to be 0.32 μg/g in mouse, a value comparable to *B. caeruleus* venom (0.13-0.23 μg/g) but higher than that of *B. candidus* and *B. multicinctus* venoms (0.04-0.13 μg/g) and lower than that of *B. fasciatus* venom (1.2-1.4 μg/g) (16). The LD_{50} reported herein is slightly higher than an earlier one reported by Chanhome *et al.* (9), which could be due to either geographic or individual variation.

B. flaviceps venom exhibited enzymatic properties similar to other *Bungarus* venoms (Table 1), including proteolytic, phosphodiesterase, alkaline phosphomonosterase, L-amino acid oxidase, acetylcholinesterase, phospholipase A, 5'-nucleotidase and hyaluronidase activities. Particularly noteworthy are its very low protease and high

acetylcholinesterase and phospholipase A_2 activities, characteristic of venoms from the genus *Bungarus* (15). Like the other *Bungarus* venoms, it did not exhibit hemorrhagic, procoagulant or anticoagulant activity *in vitro*.

Table 1. Enzymatic properties of *Bungarus flaviceps* and some other *Bungarus* venoms

Species	PRO	PDE	PME	LAAO	ACE	PLA	NUC	HYA
B. flaviceps	0.7	10	1	60	72	968	1.6	62
B. caeruleus	0.3-0.8	1-2	2-8	69-228	26-43	665-1097	1.1-2.1	332-406
B. candidus	0.5-0.7	1-2	2-5	171-292	42-85	339-732	trace	418-544
B. multicinctus	0.2-0.4	1-3	4-13	20-89	14-30	208-333	trace	59-277
B .fasciatus	0.3-0.5	4-6	2-3	65-176	12-36	297-379	3.4-3.6	4-33

PRO: protease (unit/mg) – one unit is equal to an increase of one absorbance unit per hour at 280 nm; PDE: phosphodiesterase (nmole/min/mg); PME: alkaline phosphomonoesterase (nmole/min/mg); LAAO: L-amino acid oxidase (nmole/min/mg); ACE: acetylcholine esterase (μmole/min/mg); PLA: phospholipase A (μmole/min/mg); NUC: 5'-nucleotidase (μmole/min/mg); HYA: hyaluronidase (NFU/mg) (NFU: National Formulary Unit). The substrates used were casein, bis-4-nitrophenyl phosphate, 4-nitrophenyl phosphate, L-leucine, acetylthiocholine, egg yolk suspension, 5'-AMP and human umbilical cord hyaluronic acid, respectively. Data for *B. caeruleus, B. candidus, B. multicinctus,* and *B. fasciatus* venoms are from Tan and Ponnudurai (7).

Both the indirect and double-sandwich ELISA procedures for *B. flaviceps* venom yielded an exponential dose-response curve at venom concentrations from 3 ng/mL to 100 ng/mL (not shown). The ELISA cross-reactions between antibodies against *B. flaviceps* venom and 28 venoms from snakes of the families Viperidae and Elapidae are shown in Table 2. The double-sandwich ELISA for *B. flaviceps* venom was highly specific: there were minimum cross-reactions between the IgG anti-*B. flaviceps* antibodies to all the venoms tested. Thus, double sandwich ELISA can be used for species diagnosis in *B. flaviceps* bite. In the indirect ELISA procedure, there were also no substantial cross-reactions between the antibodies and the various snake venoms tested, except for some venoms from the same genus, *Bungarus*, and the genus *Notechis*. It is interesting to note that venom from the Australian elapid, *Notechis scutatus*, yielded a very high level of indirect ELISA cross-reactions (45%) with anti-*B. flaviceps*, while venoms of *B. candidus* and *B. multicinctus* yielded only low cross-reaction proportions (5-9%), indicating that *B. flaviceps* venom shares more common epitopes with *N. scutatus* venom than with the two venoms from the same *Bungarus* genus. This is another illustration of how venoms from unrelated

snakes may share common antigens (17). A surprising feature is the total lack of indirect ELISA cross-reactions between anti-*B. flaviceps* and venoms of *Naja*, *Ophiophagus hannah* and *Enhydrina schistosa* under the experimental conditions. Both *B. flaviceps* venom and these elapid venoms contain high phospholipase A_2 content (8). While prior investigations of the immunological relationships presented by snake venom phospholipase A_2 indicated that elapid phospholipases A_2 were antigenically similar (18), our results suggest that the major *B. flaviceps* phospholipase A_2 may exhibit unique antigenic characteristics that differ substantially from venom phospholipases A_2 from the other elapids including *Naja*, *O. hannah* and *E. schistosa*.

Table 2. Cross-reactivity between antibodies to *Bungarus flaviceps* venom and various snake venoms in the indirect and double-sandwich ELISA of the venom

Venom	**Absorbance as % of *B. flaviceps* venom (mean ± SD, n = 6-9)**	
	Indirect ELISA	**Double-sandwich ELISA**
Bungarus flaviceps	100	100
Family Viperidae		
Agkistrodon p. piscivorus	0	0
Calloselasma rhodostoma	0	0
Bothrops asper	4.3 ± 0.8	0
Bothrops atrox	2.9 ± 0.5	0
Crotalus adamanteus	4.1 ± 0.2	0
Crotalus atrox	0	0
Sistrurus c. tergeminus	0	0
Protobothrops flavoviridis	0	0
Cryptelytrops albolabris	0	0
Cryptelytrops purpureomaculatus	0	0
Parais sumatranus	5.0 ± 0.7	0
Popeia popeorium	0	0
Tropidolaemus wagleri	0	0
Echis carinatus	0	0
Vipera a. ammodytes	0	0
Daboia r. siamensis	0	0
Family Elapidae		

Bungarus candidus	8.6 ± 1.2	0
Bungarus caeruleus	17.6 ± 2.2	1.7 ± 0.2
Bungarus multicinctus	5.1 ± 2.0	0
Bungarus fasciatus	18.4 ± 0.3	4.3 ± 0.6
Naja sputatrix	0	0
Naja kaouthia	0	0
Ophiophagus hannah	0	0
Dendroaspis angusticeps	0	0
Notechis scutatus	45.3 ± 1.5	1.5 ± 1.1
Notechis ater ater	6.2 ± 2.4	ND
Hoplocephalus stephensis	0	0
Enhydrina schistosa	0	0

The indirect ELISA mixture contained 100 μL of venom (25 ng/mL), 100 μL of anti-*B. flaviceps* venom (1:10000) and 100 μL of conjugate (1:4000). The absorbance at 492 nm for *B. flaviceps* venom was 1.33 ± 0.08 with anti-*B. flaviceps* venom. The double-sandwich ELISA mixture contained 100 μL of IgG anti- *B. flaviceps* venom (2 μg/mL) and 100 μL of conjugate (1:2000). Absorbance at 492 nm was 0.728 ± 0.03. ND: not determined.

Laboratory-prepared monospecific rabbit anti-*B. flaviceps,* anti-*B. candidus* and the commercial NIPM bivalent elapid antivenom were effective in neutralizing the lethal effect of the venom in mice; the amounts of *B. flaviceps* venom neutralized by these three antisera were 1600 μg/mL (167 LD_{50}'s per mL), 400 μg/mL (42 LD_{50}'s per mL) and 6667 μg/mL (695 LD_{50}'s per mL), respectively, for anti-*B. flaviceps*, anti-*B. candidus* and NIPM bivalent elapid antivenom, which was known to neutralize *B. multicinctus* venom effectively (19). The other two laboratory-prepared monospecific rabbit antisera (anti-*B. fasciatus* and anti-*N. scutatus*) and three commercial Thai Red Cross Society antivenoms (king cobra antivenom, Thai cobra antivenom and banded krait antivenom) all failed to protect the mice even when 200 μL of the antiserum was injected per mouse. Our results, however, are in contrary to that of Chanhome (9), who reported that the Thai Red Cross Society antivenom developed against banded krait (*B. fasciatus)* could effectively neutralize *B. flaviceps* venom. The reason for this discrepancy is not clear but it could be due to geographic/individual variation.

It is interesting to note that in the indirect ELISA, both *B. fasciatus* and *N. scutatus* venoms cross-reacted strongly (18%) with anti-*B. flaviceps*, while both *B. candidus* and *B. multicinctus* venoms yielded rather low cross-reaction levels (< 9%). This lack of correlation between antigenic similarity and neutralizing capacity of snake venoms

has been reported by many other authors (16). These data also suggest that the major lethal toxins of *B. flaviceps* venom are similar to those found in *B. multicinctus* and *B. candidus* venoms, that is, polypeptide neurotoxins and phospholipase A_2 toxins (8, 20). Khow *et al*. (2) have indeed shown that the major lethal toxin of *B. flaviceps* venom was similar to β-bungarotoxin from *B. multicinctus* venom.

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